Oscillatory Ca^{2+} Signals Evoked by Cholinergic Stimulation in Isolated Rat Pancreatic Duct Cells

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ABSTRACT

Acetylcholine has been known to potentiate secretin-stimulated bicarbonate secretion by the pancreatic duct system. Recent studies have shown that this phenomenon is at least partially attributable to the agonist-induced elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in pancreatic duct cells. In order to define spatiotemporal dynamics of these $[\text{Ca}^{2+}]_i$ signals, short segments of intralobular and interlobular ducts were isolated from the rat pancreas and examined by digital image microscopy using a calcium-sensitive, fluorescent probe, Fura-2. In accord with previous reports by microspectrofluorometry, duct segments stimulated with carbachol consistently displayed $[\text{Ca}^{2+}]_i$ rises, which were specifically suppressed by atropine. Present image analyses additionally demonstrated a fine fluctuate unevenness of the $[\text{Ca}^{2+}]_i$ level in the duct epithelium during the stimulation, suggesting an asynchronous oscillatory mode of response of the pancreatic duct cells.

The pancreatic duct system is densely innervated with cholinergic fibers as demonstrated histochemically (4, 18). Experiments in animals have suggested that acetylcholine released from the fibers potentiates secretin-stimulated bicarbonate secretion by the ducts (1, 3, 9). Recent studies using isolated duct segments have shown the presence of certain types of muscarinic receptors in the specimens (13), and transient elevations in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of duct cells stimulated with carbachol (6, 14, 20, 22). These findings indicate that the cholinergic potentiation of the ductal secretion is at least partially attributable to a direct effect mediated

by a receptor-dependent increase in $[\text{Ca}^{2+}]_i$ of the duct cells.

Recent techniques with digital image microscopy have disclosed regular patterns of intracellular and intercellular propagation of agonist-evoked $[\text{Ca}^{2+}]_i$ waves in some secretory epithelia, such as pancreatic acini (10, 11, 15, 21, 25), and acini and ducts of submandibular glands (24, 27). These phenomena have been presumed to contribute to the polarized release of secretory products in individual cells as well as coordinate activities of the secretory cells. In the excretory ducts of the pancreas, however, little is known concerning spatial and temporal aspects of intracellular calcium dynamics (20), probably because of difficulties in effectively isolating duct segments, which occupy no more than 4% of the total volume of this organ (2).

In the present study, segments of ducts were isolated from the rat pancreas by a modification of the collagenase digestion procedure by Hootman and Logsdon (12). These specimens were

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loaded with an intracellular calcium indicator Fura-2, and subsequently observed by fluorescent microscopy in order to define spatiotemporal properties of intracellular Ca^{2+} signals evoked with carbachol in the duct cells.

MATERIALS AND METHODS

Preparation of isolated duct segments

Male Wistar rats, 40–42 days old, were used in the present study. After an overnight fasting, the animals were sacrificed by cervical dislocation and exsanguination. The pancreas, except the tail portion, was excised and treated with purified collagenase (CLSPA, Worthington Biochemical, Freehold, NJ, U.S.A.) according to the method by Hootman and Logsdon (12), with minor modifications. Briefly, the pancreatic tissue was injected with 4.0 mL of the collagenase solution (70 units/mL) containing 0.01% trypsin inhibitor (Type 1-S, Sigma Chemical, St. Louis, MO, U.S.A.). After 60-min digestion, the tissue was mechanically disrupted with a glass pipette, rinsed twice, and resuspended in 3.0 mL of fresh HEPES-buffered physiological salt solution (PSS). The PSS contained (in mM): NaCl 118, KCl 4.7, CaCl_2 1.25, MgCl_2 1.13, NaH_2PO_4 1.0, D-glucose 5.5, and HEPES 10. This solution was supplemented with 2 mg/mL bovine serum albumin and Eagle's minimal essential medium amino acids, and adjusted to pH 7.4 with NaOH.

The dispersed pancreatic tissue was examined in a plastic Petri dish with a dissecting microscope (SZH, Nikon, Tokyo, Japan). Fragments of ducts were identified by the cobblestone-like appearance of their epithelial lining as reported by Hootman and Logsdon (12), and transferred to a separate Petri dish containing fresh PSS. The selected duct fragments were dissected under the microscope with fine forceps and iridectomy scissors in order to remove acini and accompanying vessels. The dissected ducts were cut into short tubules about 1.5 mm long with a razor blade, and again digested in 2 mL of the collagenase solution for 40 min. Segments of intralobular and fine interlobular ducts ranging in diameter from 30–100 μm were obtained in this manner, and subjected to an image analysis of their calcium signals. For morphological evaluation, a part of the isolated duct segments were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed with 1% OsO_4 , and embedded in Epon 812 according to a conventional method. Ultrathin

sections were examined with a transmission electron microscope (H-7100, Hitachi, Tokyo, Japan) after triple staining with tannic acid, uranyl acetate, and lead citrate.

Image analysis of $[\text{Ca}^{2+}]_i$

Isolated duct segments were loaded with 20 μM of Fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) for 60 min at 37°C, thoroughly rinsed in PSS, and attached to a glass coverslip with Cell-Tak (Collaborative Research, Bedford, MA, U.S.A.). The coverslip with adherent cells was mounted in the bottom of a chamber connected with a superfusion reservoir, which allowed the sequential application of different solutions onto the specimen. The PSS with above-described composition was superfused during fluorescence measurements. Carbachol dissolved at 3 μM or 30 μM in the PSS was used as a stimulant. Atropin sulfate, 30 to 300 μM in concentration, was added to the carbachol solutions for the blocking test.

Duct segments incubated with Fura-2 were examined by fluorescent microscopy. The specimen, mounted in a chamber, was placed on the stage of a Nikon Diaphot inverted microscope equipped with a 20 $\times$ epifluorescence objective. Fluorescent images at an emission wavelength of 510 ± 10 nm were captured with a silicon intensified target camera (Hamamatsu Photonics, Hamamatsu, Japan) by alternatively directing excitation wavelengths of 340 and 380 nm onto the specimen. Successive pairs of digital images were recorded at 5 or 10 sec intervals, and analyzed by an image processor (Argus 100, Hamamatsu Photonics). The ratio of the fluorescence intensity of cells excited at 340 nm and 380 nm (F340/F380) was used as an indicator of $[\text{Ca}^{2+}]_i$ according to Grynkiewicz *et al.* (8).

RESULTS

Morphological evaluation of isolated duct segments

Epithelium of isolated duct segments measuring 30–100 μm in diameter primarily consisted of principal cells, about 5 μm wide and 10 μm high (Fig. 1A). Solitary endocrine cells only occasionally intermingled among the cells. The principal cells displayed typical features as reported previously by transmission electron microscopy (5): short apical microvilli, fine infoldings of the

Fig. 1A. Cross section of an interlobular duct isolated from pancreatic tissue by collagenase digestion combined with mechanical dissection. Blood capillaries in the connective tissue which surrounds the duct have collapsed (asterisks). L: ductal lumen. Bar = 10 μ m.

Fig. 1B. High magnification of duct cells in A. Arrows indicate intercellular junctions. Collagen fibrils are dispersed among flattened fibroblasts in the periductal connective tissue (arrowheads). Bar = 2 μ m.

baso-lateral plasma membrane, scanty organelle except for relatively numerous mitochondria (Fig. 1B). Intercellular spaces between adjoining duct cells were sealed by a junctional complex on the apical side. A continuous basal lamina extended along the basal aspect of the duct epithelium.

The isolated ducts were enveloped by a connective tissue layer measuring 5–10 μ m in thickness. The connective tissue contained sparse flattened fibroblasts and collapsed blood capillaries. No smooth muscle cells were associated with the

vessels. Some connective tissue cells displayed a pyknotic nucleus, suggesting damage by collagen digestion or by mechanical dissection. Collagen fibrils, which had formed tight bundles in intact periductal connective tissue, were fragmented and dispersed among the cells.

Ratiometric images by fluorescent microscopy

Carbachol solutions at 3 μ M and 30 μ M induced $[Ca^{2+}]_i$ rises in 8 out of 9, and 12 out of 13 duct segments examined, respectively. Correlative observations of ratiometric images and transmitted light images of the isolated tissue pieces showed that central zones corresponding to the duct epithelium displayed the carbachol-evoked signals, while connective tissue limbs on both sides of the specimens were devoid of any response (Figs. 2–5). The $[Ca^{2+}]_i$ signals detected from the duct areas pursued a roughly similar time course; the $[Ca^{2+}]_i$ level as expressed by the ratio of two different fluorescence intensities (F340/F380) remained at a base line 10 sec after initiation of superfusion with the stimulant, and subsequently displayed a rapid increase to attain a plateau level 40–60 sec after initiation of the stimulation. Removal of carbachol from the superfusing medium or application of atropine sulfate 300 μ M caused a rapid return to the basal $[Ca^{2+}]_i$ level (Figs. 3 and 5).

Each duct segment examined revealed two types of regional differences in its response to carbachol. One concerns patchy regions expressing relatively low plateau levels (Figs. 2B, 3B and 4). These regions frequently overlapped with blood capillaries coursing along the bottom of the chamber, suggesting a diminution of the net amplitude of the $[Ca^{2+}]_i$ response by these inactive cell elements. The other type of regional difference corresponds to a finer fluctuate unevenness in $[Ca^{2+}]_i$ which emerged along the entire extent of the duct area during stimulation (Figs. 2C, 3C, 4 and 5C). This fluctuation was generated by numerous small spots, each of which displayed repetitive $[Ca^{2+}]_i$ spikes at a frequency of about 3 times per minute. These spots fired one after another during the excitatory phase, resulting in circulatory movements of the maximum points of the $[Ca^{2+}]_i$ level. The oscillating spots were densely disseminated in the duct area at intervals ranging from 5 to 10 μ m.

Fig. 2A-C. Time course of carbachol-induced $[Ca^{2+}]_i$ dynamics in an isolated pancreatic duct segment as shown by the transmitted light image in A. Traces a-d in graph B are recorded from corresponding squares in A. Traces 1-4 in graph C represent points 1-3 in the square c and point 4 in the square d, respectively. Each square, measuring $140 \mu m^2$ in area, can contain about five duct cells viewed in a basal aspect, while each point, $15 \mu m^2$ in area, roughly corresponds to half of the cell size.

DISCUSSION

Previous studies by microspectrofluorometry have shown carbachol-evoked $[Ca^{2+}]_i$ rises in isolated duct fragments of the pancreas in rats and guinea pigs (6, 14, 22). The present report is the first demonstration of the $[Ca^{2+}]_i$ dynamics by digital image analysis in corresponding duct segments in the rat pancreas, *i.e.*, intralobular ducts and relatively fine interlobular ducts. Our specimens prepared by a modification of the method by Hootman and Logsdon (12) appear comparable to those reported previously in such points as the integrity of the duct epithelium, preservation of the fine structure of duct cells, and susceptibility of the specimens to carbachol. The calcium waves observed by the present ratiometric imaging are compatible with those previously recorded by microspectrofluorometry with regard to the gross time course consisting of an initial rapid increase and a subsequent plateau, consistent recovery to the base line by removal of a

stimulant, and specific suppression by a muscarinic-receptor antagonist, atropine.

Microspectrofluorometry in a restricted region appears insufficient to locate the origin of the calcium signals in isolated duct segments, since the specimens are inevitably contaminated with certain amounts of connective tissue containing heterogeneous cell elements (12, 23). The present image analyses have characterized principal cells which secrete bicarbonate in the pancreatic duct system as generators of the carbachol-evoked calcium signals; $[Ca^{2+}]_i$ response within a consistent time course was detected by fluorescent microscopy from the area corresponding to duct epithelium in which principal cells predominated. Recent electrophysiological experiments have shown the Ca^{2+} -activated Cl^- current across the luminal membrane of pancreatic duct cells (14, 17). The cholinergic regulation of ductal secretion is assumed to involve the $[Ca^{2+}]_i$ increase in the principal cells and the subsequent opening of the luminal Cl^- channels, which together with the

Fig. 3A-C. Time course of $[Ca^{2+}]_i$ dynamics in an isolated pancreatic duct segment temporarily superfused with carbachol. Traces a-d in graph B are recorded from corresponding squares in the transmitted light image A. Traces 1-4 in graph C represent points 1-3 in the square b and point 4 in the square d, respectively.

luminally-located Cl^-/HCO_3^- antiport ensure recirculation of Cl^- across the luminal membrane and net secretion of HCO_3^- into the duct lumen.

Our temporal analyses of ratiometric images have shown asynchronous $[Ca^{2+}]_i$ oscillations in duct cells stimulated with carbachol; the duct cell size, about 5 μm in diameter in the basal aspect, well explains the dense distribution of the spots displaying discrete $[Ca^{2+}]_i$ pulsation in the duct area. Migration of maximum points of the $[Ca^{2+}]_i$ level in the duct epithelium may represent intercellular propagation of the oscillatory signals with a certain delay via gap junctions, which have been known to occur in the exocrine pancreas (7). Such fine oscillatory responses of duct cells have been neglected by previous microspectrofluorometry studies (14, 22); a pinhole diaphragm which was stopped down to an optical diameter of 10 μm in those experiments appears insufficient to optically isolate a single duct cell. Observations with higher spatial and temporal resolution will be required to define the calcium oscillation in the pancreatic

duct cells.

Pancreatic acinar cells stimulated with pharmacological concentrations of secretagogues, such as CCK at 100 pM or higher, or carbachol at 10 μM, have been known to display a rapid $[Ca^{2+}]_i$ rise to a peak value followed by a plateau phase (26). Digital image analyses by Habara and Kanno (10) have shown that such pharmacological or toxic stimulation evokes a sustained maximum response at the $[Ca^{2+}]_i$ level in the entire pancreatic acinus. However, the duct cells observed in the present study exhibited asynchronous oscillations of $[Ca^{2+}]_i$ in response to maximal or supramaximal stimulation with carbachol (3-30 μM), resembling pancreatic acini stimulated with lower or physiological concentrations of agonists (10). Previous investigators proposed two possible generator sites for $[Ca^{2+}]_i$ oscillations in pancreatic acinar cells: the inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} pool which periodically releases Ca^{2+} due to the bell-shape dependence of its IP_3 receptor activity on the $[Ca^{2+}]_i$ level (16), and the G

Fig. 4. Frames from a series of consecutive ratiometric images of the isolated duct segment in Fig. 3. Pseudocolor coding. The specimen was superfused with $3 \mu\text{M}$ carbachol from the time 60 sec to 180 sec. Note that points of the maximum $[\text{Ca}^{2+}]_i$ level in the duct area migrate during the stimulation.

protein-coupled muscarinic receptor complex which episodically produces IP_3 based on a feedback mechanism dependent on regulators of G protein signaling (RGS proteins) (for a review see: 19). As for the pancreatic duct cells, distinct properties of their $[\text{Ca}^{2+}]_i$ oscillations as shown in the present study may indicate certain modifications in the above-mentioned signaling pathways. Physiological and biochemical evaluation of these phenomena will help refinement of the mechanism of the $[\text{Ca}^{2+}]_i$ oscillation and elucidation of its functional significance.

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Fig. 5A-C. Time course of $[Ca^{2+}]_i$ dynamics in a pancreatic duct segment superfused with carbachol and subsequently with a mixture of carbachol and atropine. Traces a-d in graph B are recorded from corresponding squares in the transmitted light image A. Traces 1-4 in graph C represent points 1-3 in the square b and point 4 in the square d, respectively.

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